

## Differential Antiproliferative Responses of Green Tea Polyphenol for Fibroblast Cell Line versus Normal Fibroblast

Dong-Wook Han<sup>1</sup>, Hak Hee Kim<sup>1</sup>, Mi Hee Lee<sup>2</sup>, Hyun Joo Son<sup>2</sup>, Hyun Sook Baek<sup>1</sup>,  
Hyun Jeong Kim<sup>1</sup>, and Jong-Chul Park<sup>1,2\*</sup>

<sup>1</sup>Department of Medical Engineering, Yonsei University College of Medicine, Seoul 120-752, Korea

<sup>2</sup>Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea

(Received April 28, 2005/Accepted May 12, 2005)

The polyphenolic compounds present in green tea show antimutagenic, anti-inflammatory and antitumorigenic effects in many cell culture systems and animal tumor models. Epidemiologic studies have also suggested that green tea consumption might be effective in the prevention of certain human cancers. In this study, the differential antiproliferative responses of green tea polyphenols (GTP) were investigated in fibroblast cell line (L-929 cells, from mouse connective tissue) and normal fibroblasts (from neonatal human dermis). GTP treatment (100  $\mu$ M for 24 h) resulted in significant ( $p < 0.05$ ) inhibition of cell proliferation and morphological alterations with decreased local attachment, but not in normal fibroblasts. Cell cycle analysis revealed that the GTP treatment resulted in an appreciable G0/G1-phase arrest of the cell cycle in L-929 cells at 100  $\mu$ M concentration, while under similar experimental conditions, no evidence of G0/G1-phase cell cycle arrest was found in normal fibroblasts at the same dose. These results suggest that antiproliferative activity of GTP may be attributed to the differential regulation of cell cycle in fibroblast cell line and normal fibroblasts, which GTP may be exploited to craft strategies for the chemoprevention and/or therapy against cancer by GTP.

**Key words:** Antiproliferative activity, Green tea polyphenols, Fibroblast cell line, Normal fibroblasts, Cell cycle arrest

### INTRODUCTION

Green tea is one of the most popular beverages in the world and a number of epidemiological studies, though inconclusive, have shown that the consumption of green tea may provide protection against a variety of cancer types.<sup>1,2)</sup> These studies suggest that polyphenols can influence tumor formation through an inhibition of various cellular processes involved in cell replication and DNA synthesis, by interfering with cell-to-cell adhesion,<sup>3)</sup> or by inhibiting some of the intracellular communication pathways required for cell division.<sup>4)</sup> Green tea polyphenols (GTP) have been shown to possess cancer chemopreventive effects in a variety of *in vitro* and animal tumor bioassay systems.<sup>1,5,6)</sup> Most of the biological responses to green tea are believed to be mediated by its major polyphenolic antioxidant constituent epigallocatechin-3-gallate (EGCG). The antioxidant potential of EGCG is believed to be far greater than that of vitamin E and vitamin C.<sup>7,8)</sup> Furthermore, it was reported that EGCG treatment resulted in a G0/G1-phase cell cycle arrest and apoptosis of human epidermoid carcinoma (A431) cells, but not of normal human epidermal keratinocytes.<sup>9,10)</sup> These studies have also shown that

nuclear factor kB was involved in the mechanism of this differential response to EGCG in cancer cells vs. normal cells. Other report has shown that epicatechin gallate, as compared to other polyphenols in tea, would be a more effective chemopreventive agent of oral carcinoma due to its differential *in vitro* cytotoxicity and apoptosis to carcinoma HSC-2 cells and normal HGF-2 fibroblasts cells from the human oral cavity.<sup>11)</sup> As described in these reports, dose-dependent differential responses of GTP in cancer cells or cell lines vs. normal cells have been used as an important tool for cancer chemoprevention. In addition to cancer-preventive effects, in recent years, the cancer-therapeutic potential of GTP is increasingly being appreciated<sup>9,12)</sup> inasmuch as the National Cancer Institute has plans to develop tea compounds as cancer chemopreventive drugs for humans.<sup>13)</sup> In the present study, the differential antiproliferative responses of GTP for two different types of fibroblasts, mouse fibroblastic cell line (L-929 cells) and normal human fibroblasts, were investigated on the basis of cell cycle analysis.

### MATERIALS AND METHODS

#### Fibroblast Cultures and Conditions

The mouse fibroblast cell line (L-929 cells from subcutane-

---

\*Corresponding author: parkjc@yumc.yonsei.ac.kr

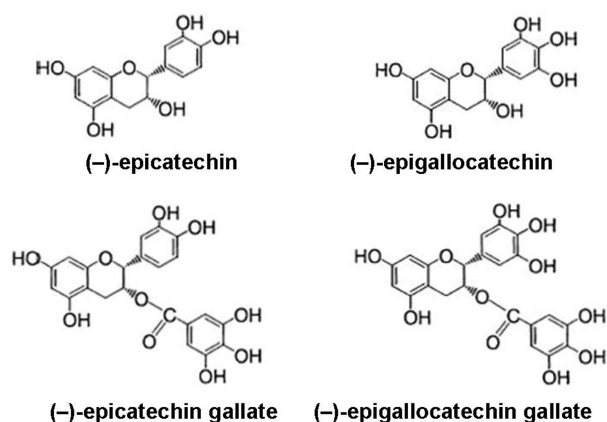
ous connective tissue) was obtained from American Type Culture Collection (Rockville, MD). The normal human dermal fibroblasts (NHDF) were prepared from neonatal human dermis using a standard procedure.<sup>14)</sup> Both fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Co., St. Louis, MO) supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (Sigma Co.). The cells were routinely maintained at 37°C and 5% CO<sub>2</sub> in a humid environment.

### Polyphenol Treatment

The polyphenolic compounds extracted from green tea were kindly supplied by Pharma Foods International Co. Ltd., Kyoto Japan. As shown in Figure 1, the mixture was mainly composed of (–)-epigallocatechin-3-*O*-gallate (28%), (–)-epigallocatechin (15.0%), (–)-gallocatechin-3-*O*-gallate (11.6%), (–)-epicatechin (7.0%), (–)-epicatechin-3-*O*-gallate (4.6%), (+)-gallocatechin (14.8%), and (+)-catechin (9.5%), and its purity exceeded 90%.<sup>15,16)</sup> In order to assess the differential antiproliferative responses of GTP for normal vs. immortalized fibroblasts, the cells were incubated for 24 h in the presence of GTP at micromolar concentrations of 0.1, 1, 10 and 100 µM, under the above-mentioned conditions, which was added to the cultured cells.

### Cell Proliferation Assay

After the cells were treated with the pre-determined concentrations of GTP, MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product] was used to estimate cell proliferation.<sup>16)</sup> The cells were incubated with 0.5 mg/ml of MTT in the last 4 h of the culture period tested at 37°C in the dark. The media were decanted and then washed twice with phosphate-buffered saline (PBS, pH 7.2). The produced formazan salts were dissolved with dimethylsulphoxide, and the absorbance was



**Figure 1.** Chemical structures of (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate, the polyphenolic constituents of green tea.

determined at 570 nm in an ELISA reader (Spectra Max 340, Molecular Device Inc., CA).

### Cell Morphology Observation

At the completion of incubation with or without GTP, cellular morphology was observed using an Olympus IX70 inverted system microscope (Olympus Optical Co., Osaka, Japan).

### Cell Cycle Analysis

For cell cycle analysis, the cells, following treatment with GTP, were collected, washed with cold PBS and resuspended in 95% cold methanol for 1 h at 4°C. The cells were then centrifuged at 1100 rpm for 5 min, and the pellet was washed twice with cold PBS, suspended in PBS and incubated with RNase (20 Units/ml, final concentration, Sigma Co.) at 37°C for 30 min. The cells were chilled over ice for 10 min, stained with 100 µg/ml propidium iodide for 1 h and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA).

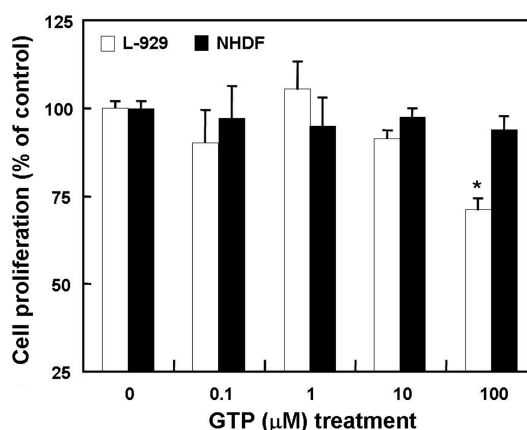
### Statistical Analysis

All the variables were tested in three independent cultures for each experiment, and each experiment was repeated twice ( $n = 6$ ). The results were reported as a mean  $\pm$  standard deviation and analyzed by Student *t*-tests. Statistical significance was considered at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Differential Effects of GTP on Proliferation and Morphology in Immortalized vs. Normal Fibroblasts

Employing the L-929 cells and NHDF, the effect of GTP on



**Figure 2.** Differential effects of GTP on proliferation in normal vs immortalized fibroblasts. The cells were incubated with GTP (0.1, 1, 10, and 100 µM for 24 h) and the cell proliferation was determined by MTT assay. The data are represented as percent of control, where the control, non-treated cells, represent 100%. The results are reported as means  $\pm$  standard deviation ( $n = 6$ ). The data is analyzed by Student *t*-tests, and the values marked with asterisks are significantly ( $p < 0.05$ ) different from the non-treated control.

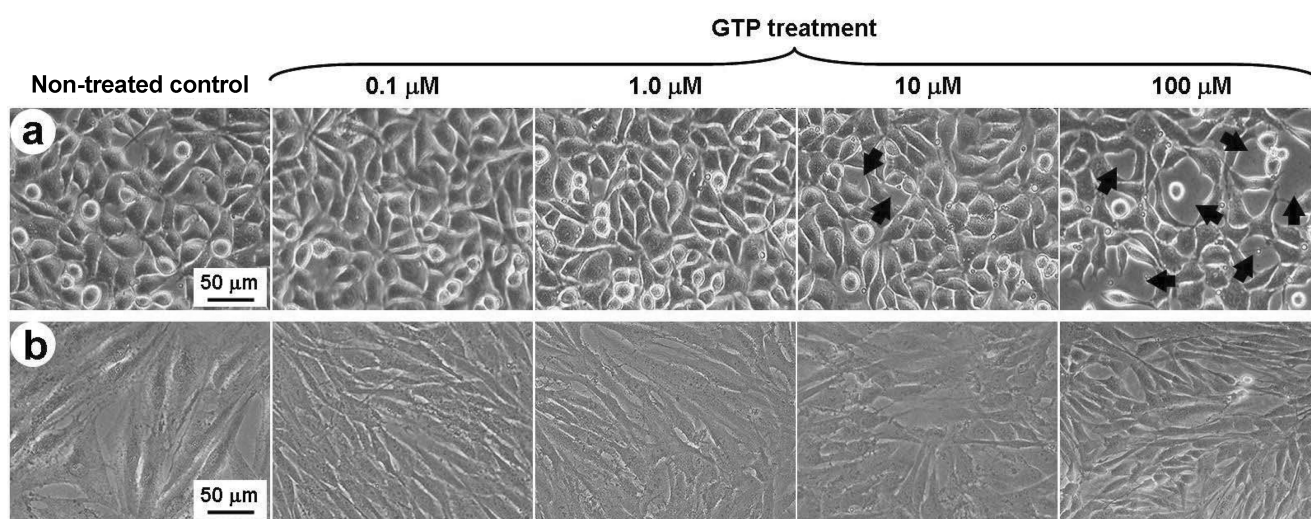
cell proliferation were examined by MTT assay. As shown in Figure 2, the GTP treatment (100  $\mu\text{M}$  for 24 h) to L-929 cells resulted in significant inhibition of the cell proliferation ( $p < 0.05$ ), but did not impart inhibitory responses for the NHDF at the same dose of GTP. On the other hand, it was revealed that the NHDF treated with over 200  $\mu\text{M}$  of GTP had partially lost their mechanism of feedback control for proliferation during the treatment but, the proliferation control was slowly recovered by the cell to cell contact and returned to a normal level after the removal of GTP from the medium (data not shown). These phenomena observed in this study might be related to the intrinsic characteristics of polyphenolic compounds. It has already been known that this compound penetrates readily into the extracellular matrix and cell membrane due to its amphipathic properties and is easily adsorbed onto any types of cellular proteins.<sup>17)</sup> Therefore, the compound combines easily with receptors on the cell surface. The adsorption of polyphenolic compounds to the protein is generated early, but the desorption rate is very slow. Moreover, some researchers reported that small ethanol concentrations (equivalent to 1 mM) inhibited cell proliferation and increased apoptosis more strongly in HepG2 cells than in normal rat hepatocytes, and 1 mM ethanol might be used as a treatment for hepatocellular carcinoma because this mainly affected tumor cells but not surrounding normal tissue.<sup>18)</sup> Others showed that the differential response of primary keratinocytes and autonomously growing keratinocyte-derived cell lines (A431 and HaCaT) to the induction of vascular endothelial growth factor by UV light could favor neoangiogenesis in the vicinity of epidermal tumor cells *in vivo*, thereby endowing them with a growth advantage over normal cells.<sup>19)</sup>

These differential antiproliferative responses of GTP for fibroblastic cell line and normal fibroblasts were also evident

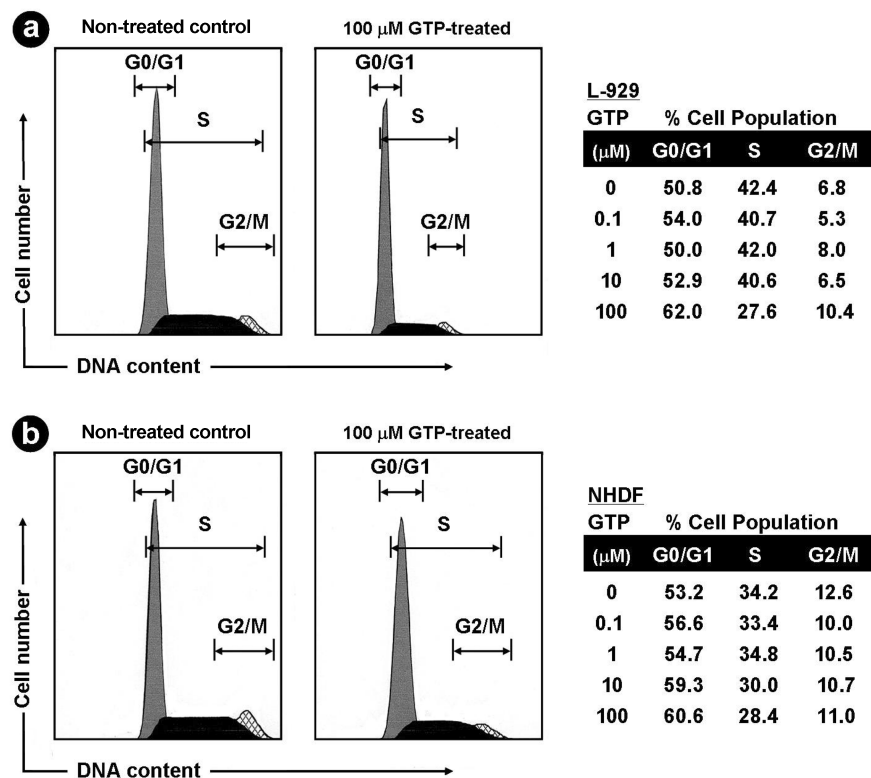
from the morphological observations (Figure 3). The non-treated control as well as the low doses (0.1–10  $\mu\text{M}$ ) of GTP did not cause any alterations in the cellular morphologies of the L-929 cells (Figure 3a). At the highest dose of GTP (100  $\mu\text{M}$ ), however, the number of attached L-929 cells were markedly decreased, indicating that the GTP treatment might result in a detachment of the cells. In contrast, this detachment phenomenon was not observed in the NHDF (Figure 3b) even at the highest dose of GTP, and the GTP itself had no effect on the cellular viability and/or survival of the cells.

#### Differential Effects of GTP on Cell Cycle Distribution in Immortalized vs. Normal Fibroblasts

To investigate the effect of GTP on distribution of the cells in the cell cycle, DNA cell cycle analysis was performed employing the growing L-929 cells and NHDF. As shown by the data in Figure 4a, the GTP treatment resulted in an appreciable increase in the G0/G1-phase of the cell cycle in the L-929 cells (50.0, 52.9 and 62.0% cells at 1, 10 and 100  $\mu\text{M}$ , respectively), with a concomitant decrease of cell population in the S-phase. Accordingly, the L-929 cells couldn't enter the S-phase during the GTP treatment and GTP might induce a G0/G1-phase arrest in the cell cycle. In this experiment, the G0/G1 cell population of controls at the specified doses did not change and ranged between 48 and 54%, probably because growing (unsynchronized) cells were employed in these experiments. Interestingly, the data also showed that 100  $\mu\text{M}$  of GTP resulted in an appreciable increase in the population of G2/M phase of the cell cycle. These results suggest that the increases in the number of the cells and DNA synthesis during the treatment period may be inhibited by GTP. There was enough evidence that the induction of a G0/G1-phase arrest in the cell cycle was due to the adsorption of polyphen-



**Figure 3.** Microscopic photographs ( $\times 200$ ) of L-929 cells (a) and NHDF (b) treated with increasing concentrations of GTP. The cells were treated with or without GTP (0.1, 1, 10, and 100  $\mu\text{M}$  for 24 h) and observed by an optical microscope. These photographs are representative images with similar results. The arrows represent locally detached sites.



**Figure 4.** Effects of GTP on cell cycle distribution in L-929 cells (a) and NHDF (b). The cells were treated with or without GTP (0.1, 1, 10, and 100  $\mu$ M for 24 h) and analyzed by flow cytometry. The percentages of cells in the G0/G1, S, and G2/M phases are calculated using Modifit computer software and represented within the histograms. The data shown at the right panel are from a representative experiment repeated six times with similar results.

nol or blocking at specific sites on the cellular membrane or DNA that effected the proliferation of cells and DNA replication.<sup>20-22)</sup> Moreover, recent study has demonstrated that expression of the metastasis-associated 67-kDa laminin receptor might confer EGCG responsiveness to cancer cells at physiologically relevant concentrations.<sup>23)</sup>

Under similar treatment conditions, the GTP treatment did not result in any significant change in the cell cycle distribution of the NHDF, only showing a slight fluctuation in the population of the phases of the cell cycle (Figure 4b). These phenomena might be due to the differential biological activities of GTP, which exhibits a strong antioxidant activity as previously described.<sup>24,25)</sup> Furthermore, it has been reported that polyphenol might play an important role in the prevention of carcinogenesis due to DNA damage by reactive oxygen radicals, as polyphenol would appear to bind to specific sites and thus interrupt the exogenous signals required for the proliferation and growth of cells.<sup>26-28)</sup>

## ACKNOWLEDGEMENTS

This study was supported by the Ministry of Health and Welfare of the Republic of Korea (Grant No. 02-PJ3-PG3-31402-0018).

## REFERENCES

1. S. K. Katiyar and H. Mukhtar, "Tea antioxidants in cancer chemoprevention," *J. Cell. Biochem. Suppl.*, **27**, 59-67 (1997).
2. R. Beliveau and D. Gingras, "Green tea: prevention and treatment of cancer by nutraceuticals," *Lancet*, **364**, 1021-1022 (2004).
3. M. Isemura, Y. Suzuki, K. Satoh, K. Narumi, and M. Motomiya, "Effects of catechins on the mouse lung carcinoma cell adhesion to the endothelial cells," *Cell Biol. Int.*, **17**, 559-564 (1993).
4. J. F. Sah, S. Balasubramanian, R. L. Eckert, and E. A. Rorke, "Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases," *J. Biol. Chem.*, **279**, 12755-12762 (2004).
5. H. Mukhtar and N. Ahmad, "Green tea in chemoprevention of cancer," *Toxicol. Sci.*, **52**, 111-117 (1999).
6. S. B. Moyers and N. B. Kumar, "Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials," *Nutr. Rev.*, **62**, 204-211 (2004).
7. C. Rice-Evans, "Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans," *Proc. Soc. Exp. Biol. Med.*, **220**, 262-266 (1999).
8. W. Bors and C. Michel, "Chemistry of the antioxidant effect of polyphenols," *Ann. NY. Acad. Sci.*, **957**, 57-69 (2002).
9. N. Ahmad, D. K. Feyes, A.-L. Nieminen, R. Agarwal, and H. Mukhtar, "Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells," *J. Natl. Cancer Inst.*, **89**, 1881-1886 (1997).

10. N. Ahmad, S. Gupta, and H. Mukhtar, "Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor  $\kappa$ B in cancer cells versus normal cells," *Arch. Biochem. Biophys.*, **376**, 338-346 (2000).
11. H. Babich, M. E. Krupka, H. A. Nissim, and H. L. Zuckerbraun, "Differential in vitro cytotoxicity of (–)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity," *Toxicol. In Vitro*, **19**, 231-242 (2005).
12. Y. Sadzuka, T. Sugiyama, S. Hirota, "Modulation of cancer chemotherapy by green tea," *Clin. Cancer Res.*, **4**, 153-156 (1998).
13. V. E. Steele, G. J. Kelloff, D. Balentine, C. W. Boone, R. Mehta, D. Bagheri, C. C. Sigman, S. Zhu, and S. Sharma, "Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays," *Carcinogenesis*, **21**, 63-67 (2000).
14. L. L. Root and G. D. Shipley, "Human dermal fibroblasts express multiple bFGF and aFGF proteins," *In Vitro Cell. Dev. Biol.*, **27A**, 815-822 (1991).
15. S.-H. Hyon and D.-H. Kim, "Long-term preservation of rat pancreatic islets under physiological conditions," *J. Biotechnol.*, **85**, 241-246 (2001).
16. Y. H. Park, D.-W. Han, H. Suh, G. H. Ryu, S.-H. Hyon, B. K. Cho, and J.-C. Park, "Protective effects of green tea polyphenol against reactive oxygen species-induced oxidative stress in cultured rat calvarial osteoblast," *Cell Biol. Toxicol.*, **19**, 325-337 (2003).
17. S.-H. Hyon and D.-H. Kim, "Hibernation of mammalian cells at a living body temperature," *Biotechnol. Bioprocess Eng.*, **6**, 289-292 (2001).
18. F. Castaneda and R. K. Kinne, "Cytotoxicity of millimolar concentrations of ethanol on HepG2 human tumor cell line compared to normal rat hepatocytes in vitro," *J. Cancer Res. Clin. Oncol.*, **126**, 503-510 (2000).
19. M. Mildner, W. Weninger, F. Trautinger, J. Ban, and E. Tschachler, "UVA and UVB radiation differentially regulate vascular endothelial growth factor expression in keratinocyte-derived cell lines and in human keratinocytes," *Photochem. Photobiol.*, **70**, 674-679 (1999).
20. T. C. Hsieh and J. M. Wu, "Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines," *Exp. Cell Res.*, **249**, 109-115 (1999).
21. Chen and L. Zhang, "The antioxidant (–)-epigallocatechin-3-gallate inhibits rat hepatic stellate cell proliferation in vitro by blocking the tyrosine phosphorylation and reducing the gene expression of platelet-derived growth factor-beta receptor," *J. Biol. Chem.*, **278**, 23381-23389 (2003).
22. C. S. Hofmann and G. E. Sonenshein, "Green tea polyphenol epigallocatechin-3 gallate induces apoptosis of proliferating vascular smooth muscle cells via activation of p53," *FASEB J.*, **17**, 702-704 (2003).
23. H. Tachibana, K. Koga, Y. Fujimura, and K. Yamada, "A receptor for green tea polyphenol EGCG," *Nat. Struct. Mol. Biol.*, **11**, 380-381 (2004).
24. D.-W. Han, Y. H. Park, J. K. Kim, K.-Y. Lee, S.-H. Hyon, H. Suh, and J.-C. Park, "Effects of green tea polyphenol on preservation of human saphenous vein," *J. Biotechnol.*, **110**, 109-117 (2004).
25. D. K. Rah, D.-W. Han, H. S. Baek, S.-H. Hyon, and J.-C. Park, "Prevention of reactive oxygen species-induced oxidative stress in human microvascular endothelial cells by green tea polyphenol," *Toxicol. Lett.*, **155**, 269-275 (2005).
26. S. K. Katiyar, F. Afaq, K. Azizuddin, and H. Mukhtar, "Inhibition of UVB-induced oxidative stress-mediated phosphorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (–)-epigallocatechin-3-gallate," *Toxicol. Appl. Pharmacol.*, **176**, 110-117 (2001).
27. S. E. Tobi, M. Gilbert, N. Paul, and T. J. McMillan, "The green tea polyphenol, epigallocatechin-3-gallate, protects against the oxidative cellular and genotoxic damage of UVA radiation," *Int. J. Cancer*, **102**, 439-444 (2002).
28. V. Lewerenz, S. Hanelt, C. Nastevska, C. El-Bahay, E. Rohrdanz, and R. Kahl, "Antioxidants protect primary rat hepatocyte cultures against acetaminophen-induced DNA strand breaks but not against acetaminophen-induced cytotoxicity," *Toxicology*, **191**, 179-187 (2003).